PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 21726WO	**FOR FURTHER ACTION** See Form PCT/IPEA/416	
International application No. PCT/EP2005/000555	International filing date *(day/month/year)* 17.01.2005	Priority date *(day/month/year)* 19.01.2004
International Patent Classification (IPC) or national classification and IPC INV. C12P13/00 C12P13/02 C12P7/40 C12P7/44 C12N1/14 C12N1/20 C12N9/04 C12N15/11 C07C229/30 C07C229/06 C07D223/10 C08L77/00 C12P7/42		
Applicant DSM IP ASSETS B.V. et al.		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 10 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 3 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in electronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16.11.2005	04.05.2006
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Thumb, W Telephone No. +49 89 2399-7350

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2005/000555

Box No. I Basis of the report

1. With regard to the **language**, this report is based on

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3(a) and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4(a))
   - ☐ international preliminary examination (under Rules 55.2(a) and/or 55.3(a))

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

   Description, Pages

   1-31 as originally filed

   Sequence listings part of the description, Pages

   1-3 as originally filed

   Claims, Numbers

   1-27 received on 17.11.2005 with letter of 16.11.2005

   ☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 24-27

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination *(specify)*:

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☒ the claims, or said claims Nos. 24-27 are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*.

see separate sheet

☐ no international search report has been established for the said claims Nos.

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rules 13*ter*.1(a) or (b) and 13*ter*.2.

☐ a meaningful opinion could not be formed without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions, and such tables were not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in electronic form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See separate sheet for further details

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2005/000555

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-23
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-23
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-23
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this report was established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ on paper

   ☒ in electronic form

   c. time of filing/furnishing:

   ☒ contained in the international application as filed

   ☒ filed together with the international application in electronic form

   ☐ furnished subsequently to this Authority for the purposes of search and/or examination

   ☐ received by this Authority as an amendment* on

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

* *If item 4 in Box No. I applies, the listing and/or table(s) related thereto, which form part of the basis of the report, may be marked "superseded."*

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

In view of the arguments of the applicant, submitted in the letter dated 16.11.2005, the Examining Authority has come to the conclusion that claims 24-27 are so insufficiently disclosed within the meaning of Article 5 PCT, that no meaningful opinion on novelty and inventive step can be given.

The applicant argues that the production of 6-ACA in document D5 will not lead to a product having a carbon isotope ratio which is distinctly different from that in environmental carbon dioxide, since the substrate used in D5 is allegedly obtained from fossil sources. Therefore, the applicant implies that the present invention refers to compounds produced by *de-novo* synthesis of the compounds of claims 24-27 in a microorganism. A general reference is made to this process on pages 11, line 19 - page 13, line 12, without identifying microorganisms, which are actually capable of synthesising e.g. 6-ACA from natural carbon sources. The example demonstrating bioconversion of 6-AHEA, starting on page 28 of the specification, do not identify the source of the substrate 6-AHEA, and also not disclose synthesis of the desired molecules starting from natural carbon sources. Therefore, the application as filed does not disclose a microorganism which is actually capable of *de-novo* synthesis of, e.g., 6-ACA from natural carbon sources, resulting in a molecule having a carbon isotope ratio corresponding to that of environmental carbon dioxide. Therefore, the subject-matter of claims 24-27 is seen as being merely an invitation to perform research, ie. identifying those microorganisms capable of performing the above-described biosynthesis, which, starting from the teaching of the specification, cannot be put into practice without undue burden.

Therefore, claims 24-27 cannot be allowed pursuing the provisions of Article 5 PCT.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The documents mentioned in the International search report are cited by the following abbreviations:

D1: WO 03/066863 A (ESAKI NOBUYOSHI ; KAMACHI HARUMI (JP); KURIHARA TATSUO (JP); SHOWA DEN) 14 August 2003 (2003-08-14)

D2: ROHDICH FELIX ET AL: "Enoate reductases of Clostridia: Cloning, sequencing, and expression" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 276, no. 8, 23 February 2001 (2001-02-23), pages 5779-5787, XP002285475 ISSN: 0021-9258

D3: SIMON H ET AL: "CHIRAL COMPOUNDS SYNTHESIZED BY BIOCATALYTIC REDUCTIONS" ANGEWANDTE CHEMIE. INTERNATIONAL EDITION, VERLAG CHEMIE. WEINHEIM, DE, vol. 24, no. 7, 1 July 1985 (1985-07-01), pages 539-553, XP000196422 ISSN: 0570-0833

D4: DATABASE CHEMABS [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; 2003, STEINBACHER, STEFAN ET AL: "Enoate reductase family" XP002285477 retrieved from STN Database accession no. 2003:101473

D5: JP 50 006776 A (TEIJIN LTD., JAPAN) 23 January 1975 (1975-01-23)

D6: CN-A-1 358 841 (YUNNAN MICROBE INST) 17 July 2002 (2002-07-17)

D7: WHELAN A ET AL: "NYLON 6 (PA6)" KUNSTSTOF EN RUBBER, WYT EN ZONEN UITGEVERS. ROTTERDAM, NL, vol. 39, no. 3, March 1986 (1986-03), pages 38-39, XP001173573 ISSN: 0167-9597

D8: MIURA K ET AL: "MOLECULAR CLONING OF THE NEMA GENE ENCODING N-ETHYLMALEIMIDE REDUCTASE FROM ESCHERICHIA COLI" BIOLOGICAL & PHARMACEUTICAL BULLETIN (OF JAPAN), PHARMACEUTICAL SOCIETY OF JAPAN, JP, vol. 20, no. 1, 1997, pages 110-112, XP009049638 ISSN: 0918-6158

D9: EP-A-0 494 078 (LEPETIT SPA) 8 July 1992 (1992-07-08)

2. The present application concerns in claims 1-14 and 21-23 a process for biochemical synthesis of 6-NH2-caproic acid using an enoate reductase.
Claims 15-20 are directed to several strains of host microorganisms cloned with the enzyme alpha,beta-enoate reductase from certain microorganisms; the feature of the strain being "for the biochemical synthesis" is not limiting.
Claims 24-27 are drafted as "product by process"-claims, which are directed to the compounds per se which consequently must fulfil themselves the requirements of, i.a., novelty and inventive step.

3. Brief discussion of the prior art documents:

D1 discloses reductase enzymes of the application, the isolated gene sequences of the said enzymes, vectors comprising the said sequence, and host cells transfected therewith; also disclosed is the reduction of alpha,betaunsaturated carbonyl compounds using the said enzymes/host cells.
D2 concerns the gene of the enoate reductase isolated from Clostridium tyrobutyricum or Clostridium thermoaceticum and its expression in E. co/i.
D3 concerns the same reaction and the same enzyme as in the present application, but does not explicitly mention 6-aminohex-2-enoic acid and 6-carboxy-6-aminohex-2-enoic acid. Table 2 shows a broad spectrum of compounds similar in structure to the ones used in the present application, and Table 3 discloses amino compounds as substrate for the same strain Clostridium tyrobutyricum DSM 1460.
D4 is a review about enoate reductases which says that this enzyme has a broad substrate specificity.
D5 discloses a biochemically produced compound as in present claim 24.
D6 discloses a biochemically produced compound as in present claim 25.
D7 discloses the production of nylon.
D8 refers to the cloning of the E.coli nemA gene and funtional assessment of the gene product.

4. Novelty and inventive step - Art. 33(2) and (3) PCT

4.1 Claims 1-23 are novel within the meaning of Article 33(2) PCT:
None of the prior art documents discloses the claimed processes, in that all documents which use the enzyme specified in said claims use structurally slightly different compounds as substrate.
The specific host cells of claims 15-20 are also not disclosed in any of the prior art documents. Therefore the subject-matter of claims 1-23 appears to be new.

4.2 Claim 1 also appears to be inventive within the meaning of Article 33(3) PCT.
Document D3, which is considered to represent the most relevant state of the art, discloses characterisation of alpha,beta-enoate reductases derived from a number of microorgamisms. In tables 2 and 3, a number of substrates having alpha,beta-unsaturated carboxylic compounds are listed.
The subject-matter of claim 1 differs from the teaching of D3 in that a different substrate is used, namely 6-AHEA, or a precursor thereof.
The underlying objective technical problem to be solved by claim 1 may therefore be seen in providing an alternative substrate for alpha,beta-enoate reductases.
The substrate of present claim 1 is an unsubstituted linear aliphatic molecule containing a primary amino group.
The arguments of the applicant referring to the nature of said substrate, already included in the specification at page 4, line 13 - page 5, line 14, are accepted by the Examining Authority. The skilled person would not appear to be encouraged to use alpha,beta-enoate reductases with a substrate containing a primary amino group due to an expected preferred hydride-transfer to this group rather than to the unsaturated double-bond.
D3 does not disclose substrates containing primary amino groups (see tables 2 and 3). D1 refers to alpha-substituted substrates (see paragraphs 12 and 23). The examples do not disclose the use of a substrate having an amino group (see example 1). D2, referring to cloning of alpha,beta-enoate reductase of clostridia, also does not describe reactions on substrates containing a primary amino group (page 5782, right-hand column).
D4 only mentions broad substrate specificity, without giving specific examples.

The other cited prior art documents do not deal with alpha,beta-enoate reductases. Therefore, none of the documents, taken alone or in combination, teaches or renders obvious that alpha,beta-enoate reductases can be used to reduce 6-AHEA in the biochemical synthesis of 6-amino caproic acid.
Claim 1 as well as claims 2-14 and 21-23 therefore appear to meet the requirements of Article 33(3) PCT.

4.3 Claims 15-20 pertain to host cells comprising cloned alpha,beta-enoate reductase genes, which are suitable for carrying out the processes according to the present invention. Said host cells are neither disclosed nor rendered obvious in the prior art and therefore meet the requirements of Article 33(3) PCT.

Re Item VIII
Certain observations on the international application

The application discloses reduction of 6-AHEA only using alpha,beta-enoate reductases derived from *Clostridium tyrobutyricum, Moorella thermoacetica*, and the nemA gene of *E.coli* K12. In view of the argument of the applicant that it would not be prima vista obvious that alpha,beta-enoate reductases can use 6-AHEA as a substrate, it would appear that claims relating to processes using or host cells containing enzymes derived from different organisms than the above referred to lack support within the meaning of Article 6 PCT.

Enclosure to letter dated 15 November 2005 concerning European Patent Appln. No. PCT/EP2005/000555; - DSM IP Assets B.V. -; ref:21726W0, clean version

REVISED CLAIMS

1. Process for the biochemical synthesis of 6-amino caproic acid wherein either 6-aminohex-2-enoic acid of formula [1] (6-AHEA)

$$H_2N - CH_2 - CH_2 - CH_2 - CH = CH - COOH \qquad [1]$$

or wherein 6-amino-2-hydroxy-hexanoic acid (6-AHHA), a compound capable of being transformed into 6-aminohex-2-enoic acid, is treated with an enzyme having α,β-enoate reductase activity towards molecules containing an α,β-enoate group and a primary amino group, in particular with an enzyme having α,β-enoate reductase activity towards 6-aminohex-2-enoic acid.

2. Process according to claim 1, characterized in that the enzyme having α,β-enoate reductase activity is an enzyme originating from a microorganism from the group of species of *Acetobacterium* sp., *Acremonium* sp., *Agrobacterium* sp., *Burkholderia* sp., *Cephalosporium* sp., *Clostridium* sp., *Escherichia* sp., *Moorella* sp., *Ochrobactrum* sp., *Pseudomonas* sp., *Salmonella* sp., *Shigella* sp., *Tilachlidium* sp., *Yersinia* sp., and *Vibrio* sp.

3. Process according to one of claims 1 or 2, characterized in that the enzyme having α,β-enoate reductase activity is an enzyme originating from *Acremonium* sp., *Clostridium* sp., *Moorella* sp. or *Ochrobactrum* sp.

4. Process according to claim 3, characterized in that the enzyme having is an enzyme from *Acremonium strictum* CBS114157, *Clostridium tyrobutyricum* DSM1460, *Moorella thermoacetica* DSM1974, *Ochrobactrum anthropi* NCIMB41200, or *Clostridium kluyveri* DSM555.

5. Process according to claim 1 or 2, characterized in that the enzyme having α,β-enoate reductase activity has aerostable α,β-enoate reductase activity and is an enzyme originating from a microorganism from the group of species of *Agrobacterium* sp., *Burkholderia* sp., *Escherichia* sp., *Pseudomonas* sp., *Salmonella* sp., *Shigella* sp., *Yersinia* sp., and *Vibrio* sp.

6. Process according to claim 5, characterized in that the enzyme having aerostable α,β-enoate reductase activity is an enzyme originating from an *Escherichia coli* species.

7. Process according to claim 6, characterized in that the enzyme having aerostable α,β-enoate reductase activity is an enzyme originating from from *Escherichia coli* K12.

Enclosure to letter dated 15 November 2005 concerning European Patent Appln. No. PCT/EP2005/000555; - DSM IP Assets B.V. -; ref:21726W0, clean version

8. Process according to any of claims 1-7, characterized in that 6-aminohex-2-enoic acid is being converted into 6-amino caproic acid at a pH in the range of from 3 to 9.

9. Process according to claim 8, characterized in that, the pH is in the range of from 4 to 8.

10. Process according to claim 9, characterized in that the pH is in the range of from 5 to 8.

11. Process according to claim 8, characterized in that, the pH is in the range of from 5.5 to 7 under anaerobic conditions and of from 6.5 to 8 under aerobic conditions.

12. Process according to any of claims 1-11, characterized in that the process is carried out in a host organism selected from the group of genera consisting of *Aspergillus*, *Bacillus*, *Corynebacterium*, *Escherichia* and *Pichia*.

13. Process according to claim 12, characterized in that the process is carried out in a host organism selected from the group of *Escherichia coli*, *Bacillus*, *Corynebacterium glutamicum*, *Aspergillus niger* or *Pichia pastoris* host organisms.

14. Process according to claim 12 or 13, characterized in that in the host organism an α,β-enoate reductase gene encoding an enzyme having α,β-enoate reductase activity towards molecules containing an α,β-enoate group and a primary amino group is cloned and expressed.

15. An *Escherichia coli* host cell wherein the α,β-enoate reductase gene from *Ochrobactrum anthropi* NCIMB41200, or from *Acremonium strictum* CBS114157 is cloned and expressed.

16. A *Bacillus* host cell wherein the α,β-enoate reductase gene from *Moorella thermoacetica* DSM1974, or from *Clostridium tyrobutyricum* DSM1460, or from *Ochrobactrum anthropi* NCIMB41200, or from *Acremonium strictum* CBS114157 is cloned and expressed.

17. A *Corynebacterium glutamicum* host cell wherein the α,β-enoate reductase gene from *Moorella thermoacetica* DSM1974, or from *Clostridium tyrobutyricum* DSM1460, or from *Ochrobactrum anthropi* NCIMB41200, or from *Acremonium strictum* CBS114157 is cloned and expressed.

18. An *Aspergillus niger* host cell wherein the α,β-enoate reductase gene from *Acremonium strictum* CBS114157, or from *Moorella thermoacetica* DSM1974, or from *Clostridium tyrobutyricum* DSM1460, or from *Ochrobactrum anthropi* NCIMB41200 is cloned and expressed.

Enclosure to letter dated 15 November 2005 concerning European Patent Appln. No. PCT/EP2005/000555; - DSM IP Assets B.V. -; ref:21726W0, clean version

19. A *Pichia pastoris* host cell wherein the α,β-enoate reductase gene from *Acremonium strictum* CBS114157, or from *Moorella thermoacetica* DSM1974, or from *Clostridium tyrobutyricum* DSM1460, or from *Ochrobactrum anthropi* NCIMB41200 is cloned and expressed.

20. A host cell selected from the group of *Aspergillus*, *Bacillus*, *Corynebacterium*, and *Pichia* host cells, in which the aerostable α,β-enoate reductase gene *nemA* from *E. coli* K12 is cloned and expressed.

21. Process for precursor fermentation of 6-amino caproic acid starting either from 6-aminohex-2-enoic acid (6-AHEA) or from 6-amino-2-hydroxyhexanoic acid (6-AHHA), and applying at least an enzymatic step with an enzyme having α,β-enoate reductase activity towards molecules containing an α,β-enoate group and a primary amino group, in particular with an enzyme having α,β-enoate reductase activity towards 6-aminohex-2-enoic acid.

22. Process according to claim 21, characterized in that the process is performed in a microorganism wherein 6-aminohex-2-enoic acid is being formed *in vivo*.

23. Process according to claim 22, characterized in that 6-aminohex-2-enoic acid is being formed *in vivo* from solutions or slurries containing a suitable carbon source.

24. Biochemically produced 6-aminohex-2-enoic acid, having a ^{12}C versus ^{13}C versus ^{14}C isotope ratio of about the same value as occurring in environmental carbon dioxide.

25. Biochemically produced 6-amino-hexanoic acid having a ^{12}C versus ^{13}C versus ^{14}C isotope ratio of about the same value as occurring in environmental carbon dioxide.

26. ε-Caprolactam produced from biochemically produced 6-aminohex-2-enoic acid or 6-amino-hexanoic acid, and having a ^{12}C versus ^{13}C versus ^{14}C isotope ratio of about the same value as occurring in environmental carbon dioxide.

27. Nylon-6 and other derivatives produced from any of the biochemically produced products of claims 24 or 25, or from ε-caprolactam according to claim 26, and having a ^{12}C versus ^{13}C versus ^{14}C isotope ratio of about the same value as occurring in environmental carbon dioxide.